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Characterization of lactic acid bacteria producing bacteriocins against chicken *Salmonella enterica* and *Escherichia coli*

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30 strains of lactic acid bacteria (LAB) isolated from 5 samples, raw and fermented cow milk, cow meat, soil and fermented-dried cassava, were screened for their antimicrobial activity against poultry pathogens *Salmonella enterica* CIP8132 and *Escherichia coli* CIP548. All the lactobacillus strains were active against the target pathogens with the strains LF2, SM3, SM4, V3 and V5 exhibiting activity higher than 25 mm. 4 of the 5 isolated cultures were confirmed as bacteriocin producing cultures, in addition LF2 strain was cocci, can grow on sucrose, was thermal resistant and was partially inhibited by proteinase K and lysozyme suggesting a nisin-like molecule. This strain with promising future needs to be further characterised.

Key words: Lactic acid bacteria, antimicrobial activity, bacteriocins, *Salmonella enterica*, *Escherichia coli*.

INTRODUCTION

During the last decade, poultry production has become an important sector of activity in Cameroon. This has contributed to increasing use of antibiotics as growth factor, which has been shown to have negative effect not only to human being, but also to animal production. In fact it is well recognized that the extensive use of antibiotics in animal production contributes to the development of antibiotic-resistant pathogens both in humans and domesticated animals. This goes a long way to increasing the mortality and decreasing poultry productivity (Wegener et al., 1997). In 1998 in European union, this problem led to the interdiction (European rule 2821/98/CEE) of antibiotics in animal feeding as growth factor. In this respect many studies have focused on bacteriocins secreted by lactic acid bacteria (LAB) to inhibit the main undesirable poultry pathogens *Salmonella enterica* and *Escherichia coli* (Pascual et al., 1999; Ashraf et al., 2005; Ma et al., 2006).

LAB are well known for their production of peptides and proteins with antimicrobial properties, known as bacteriocins. The potential applications of LAB bacteriocins in the food and health care sectors have attracted the strong interest of academia and the industry, resulting in an impressive amount of published research on their production, purification, genetics and applications (Papagianni, 2003). In the future, these substances could replace antibiotics usually used to prevent diseases and promote growth of animals in farming.

Bacteriocins are proteinaceous agents with antimicrobial activity against species closely related to the producer strain (Tagg et al., 1976). Bacteriocin producing LAB have the “generally recognized as safe” (GRAS) status and can be administrated to animals to strengthen the barrier function of the gut microflora and/or for a non specific enhancement of the immune system (Tome et al., 2008). In this respect Nitisimprasert et al. (2000) have studied the antimicrobial activity of LAB from chicken intestine against the antibiotic resistant pathogenic strains of *E. coli* and *Salmonella* sp. Identification of 3 effective thermo-tolerant LAB were classified as *Lacto-bacillus reuteri* strains KUB (AC, KUB-AC16 and KUB-AC20). These isolates displayed broad inhibition spec-trum against *E. coli* and *Salmonella* sp. resistant to va-rious antibiotics by exerting bacterium like activity. In adi-

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addition, Sukyai (2003) indicated that the main products from carbohydrate fermentation by *L. reuteri* KUB-AC5 were lactic and acetic acids. This author equally reported that the antimicrobial activity of *L. reuteri* KUB-AC5 resulted from synergistic activities of acids and proteinaceous substances. Since sources and activity of bacteriocin producing LAB varied with ecosystems, it is likely that number of studies have to be made world while in order to discover new and active LAB.

In the foregoing the aim of this study was to determine the activity of LAB strains isolated from raw and fermented cow milk, cow meat, soil and dried cassava against 2 selected poultry pathogens *S. enteric* and *E. coli.* Particular interest was given to active bacteriocin secreting LAB strains.

MATERIALS AND METHODS

Isolation and characterization of lactic acid bacteria

The test strains isolated from crude cow milk, local fermented cow milk, fresh cow meat and fresh cassava were obtained from a local market in Ngaoundere. Soils samples were collected from cereals-grinding places at the local markets. Fermented milk was directly submitted to isolation of LAB strains while the other samples were first submitted to treatments prior to isolation. The fresh cow and cassava samples were submitted to 24 h natural fermentation while the soil samples were added to peptone water and incubated for 24 h at 37°C before isolation. For the isolation of LAB, surface plated MRS agar (Oxoid, Hamp-shire, UK) were incubated for 72 h at 37°C. The colonies were randomly picked from plate and purified by successive streaking on MRS agar media before being subjected to characterization. Gram-positive and catalase-negative rods were isolated and charac-terized by phenotypic criteria (Harrigan and McCance, 1990). The isolates were stocked on MRS agar slant at 4°C and sub-cultured monthly.

Test micro organisms

Food borne pathogens used for testing antibacterial activity were *S. enterica* CIP8132 and *E. coli* CIP548 received from the collection of microbiology laboratory of the national school of agro-industrial sciences (university of Ngaoundere, Cameroon). They were sub-cultured 3 times in TSB (Tryptone Soy Broth) (Oxoid, Hampshire, UK) for 24 h for activation prior to experimental use.

Antimicrobial activity of isolated LAB

Antimicrobial activity of isolated LAB strains was carried out by a well diffusion method as described by Tagg and McGiven (1971). Briefly, wells (10 mm of diameter) in MRS agar were incubated with bacteriocinogenic culture of LAB for 48 h. After this, the plates were overlaid with a solution of target strains (*E. coli* or *S. enterica*) obtained by mixing 50 µl of strain (24 h culture on TSB broth at a concentration of 10^8 cfu/ml) with 200 ml of PCA (plate count agar) (Oxoid, Hampshire, UK) . After the overlays had solidified, the plates were incubated for 24 h and then examined for a zone of inhibition around the well. The activity representing the diameters of the inhibition zone was expressed in mm.

Physiological and biochemical characterizations of selected LAB

All isolates active against *S.enterica* and *E. coli* were initially tested for Gram reaction, catalase reaction by the 3% H_2O_2 method and cell morphology using phase contrast microscopy.

The strains were then characterised by their carbohydrate fermentation pattern using the API 50 CH strips and the API 50 CHL medium according to the manufacturer's instructions (API system, Bio-Merieux, France). Strains were tentatively identified into species using APIDENT (version 2.0, Bio-Merieux).

The strains were further tested for salt tolerance incubation for 48 h at 37°C in MRS broth supplemented with 6.5% NaCl.

The Growth at different temperatures was observed in MRS broth after incubation for 48 h at 8, 37 and 45°C. The determination of their fermentative type was also done in MRS broth with Durham bell.

Partial characterization of inhibitory substances in supernatant

Selected LAB strains were grown in MRS broth for 48 h at 37°C and the cell-free supernatant (CFS) was obtained by vacuum filtration (Sartorius, 0.45 µm). Some filtrates were neutralized with 3 M NaOH to pH 7.0 to eliminate the action of acid. Unneutralised and neutralised filtrates were then used for the antimicrobial activity using the agar well diffusion method. In this respect 50 µl of each pathogenic strain culture at a concentration of 10^8 cfu/ml was suspended in 200 ml PCA agar and poured into petri dishes. When the agar solidified, a 300 µl of the CFS was filled in 10 mm diameter sealed wells which were cut in the nutrient agar. The plates were then incubated at 37°C for 24 h after which the diameter of inhibition was determined.

In order to test the sensitivity of CFS inhibitory substances to proteinase K and lysozyme (Eurobio, France), CFS were incubated for 24 h with enzymes (1mg/ml final concentration) in a 10 mM potassium phosphate buffer pH 7 at 37°C. The treated and untreated CFS samples were then tested for their antibacterial activity using the well diffusion method as described above.

The heat sensitivity was determined by heating aliquots of CFS preparation (5 ml) at 60, 80 and 100°C for 15 min prior to antibacterial activity evaluation.

Statistical analysis

Means and standard deviations were calculated from 3 independent replicate trials and subjected to analysis of variances using SPSS Statistical package (Statsoft, 1995). Differences between means were evaluated using Duncan multiple range test.

RESULTS AND DISCUSSION

Lactobacilli strains

Totally, 30 strains from the 5 ecosystems were Gram-positive and catalase- negative and were characterized morphologically (Table 1). It can be seen that fermented milk was the best substrate for LAB with 16 LAB strains isolated while cassava was the least with 2 LAB strains. The potential of milk as LAB substrate had been demonstrated in an earlier study on traditional fermented milk in Burkina Faso which reported 98 LAB strains over 100 strains isolated (Aly et al., 2004). Presently lactobacilli isolates from MRS agar were classified as *Lactobacillus* sp. (26 strains) based on its rod shape while others can be classified as *Streptococcus,* based on their spherical shape.

Table 1. Morphology of isolated LAB strains.

Ecosystem	Strain	Identification	Shape
Raw cow milk	LF2	-	Spherical
	LF5	*Lactobacillus* ssp.	Straight rods
	LF8	*Lactobacillus* ssp.	Straight rods
Fermented milk	Ki1	*Lactobacillus* ssp.	Straight rods
	Ki2	*Lactobacillus* ssp.	Straight rods
	Ki3	*Lactobacillus* ssp.	Straight rods
	Ki4	*Lactobacillus* ssp.	Straight rods
	Ki5	*Lactobacillus* ssp.	Straight rods
	Ki6	*Lactobacillus* ssp.	Straight rods
	Ki7	*Lactobacillus* ssp.	Straight rods
	Ki8	*Lactobacillus* ssp.	Straight rods
	Ki9	*Lactobacillus* ssp.	Straight rods
	Ki10	*Lactobacillus* ssp.	Straight rods
	Ki11	*Lactobacillus* ssp.	Straight rods
	Ki12	*Lactobacillus* ssp.	Straight rods
	Ki13	*Lactobacillus* ssp.	Straight rods
	Ki14	*Lactobacillus* ssp.	Straight rods
	Ki15	*Lactobacillus* ssp.	Straight rods
	Ki16	*Lactobacillus* ssp.	Straight rods
Cow meat	V2	-	Spherical
	V3	*Lactobacillus* ssp.	Straight rods
	V4	*Lactobacillus* ssp .	Straight rods
	V5	*Lactobacillus* ssp	Straight rods
Dry cassava	MaS1	-	Spherical
	MaS4	-	Spherical
Soil samples	SM3	*Lactobacillus* ssp.	Straight rods
	SM4	*Lactobacillus* ssp.	Straight rods
	SM5	*Lactobacillus* ssp.	Straight rods
	SM6	-.	Spherical
	SM7	*Lactobacillus* ssp	Straight rods

Antimicrobial activity of isolated LAB

All the LAB strains were found to produce inhibition zones against pathogenic bacteria *S. enterica* CIP8132 and *E. coli* CIP548 (Table 2). Irrespective of the patho-gens, it was observed that the antimicrobial activity vary significantly ($P < 0.05$) from one strain to another with the strains LF2 and SM3 producing the highest inhibition zones against *S. enterica* while LF2, V3, V5 SM4 and SM3 exhibited the highest activity against *E. coli.*

The physiological and biochemical characteristics of the selected LAB strains LF2, V3, V5, SM3 and SM4 are shown in Table 3. The results revealed that all the 5 lactic strains are mesophilic homo- fermentative. 3 strains, SM3, SM4 and V3, were identified as *Lactobacillus brevis* 1. *L. brevis* 1 had been isolated from raw goat milk and cha-racterised as mesophilic hetero-fermentative (Bettache and Mebrouk, 2004). The carbohydrate fermentation pat-tern of a strain has been suggested to vary with the eco-system (Shea, 2004). In this respect *L. brevis* 1 may exhibit homo or hetero fermentative behaviour according to its environment. Further investigations using molecular techniques need to be performed in order to complete the identification of these strains.

Partial characterization of inhibitory substances in supernatant

As shown in Figure 1, the antimicrobial activities of selected LAB-CFS are significantly influenced by pH. In this respect, it was observed that the activities at pH 4.5 were significantly higher than those at pH 7.0 suggesting an inhibition effect of acidity on the growth of *S. enterica* CIP8132 and *E. coli* CIP548 pathogens. In fact most of LAB excretes acid that has been shown to inhibit growth of pathogen. On the other hand, it was observed a residual activity at pH 7.0 suggesting that compounds other than acids inhibit the growth of *S. enterica* CIP8132 and *E. coli* CIP548. These observations are in agreement with

Table 2. Diameter of zone of inhibition (mm) of isolated LAB against test micro organisms.

Ecosystem	LAB strains	Test microorganisms	
		S. enterica	***E. coli***
Raw cow milk	LF2	30.0 ± 1,4 a	39.0 ± 1,4 a
	LF5	20.0 ± 0,7 cd	25.0 ± 0,7 c
	LF8	18.0 ± 0,7 de	20.0 ± 0,7 efg
Fermented milk	Ki1	20.0 ± 1,4 cd	16.0 ± 1,4 hi
	Ki2	18.0±1,4 de	24.0 ± 1,4 cd
	Ki3	14.0 ± 0,0 fg	16.0 ± 0,0 hi
	Ki4	16.0 ± 0,0 ef	20.0 ± 0,0 efg
	Ki5	14.0 ± 0,0 fg	19.0 ± 0,0 fg
	Ki6	14.0 ± 0,0 fg	18.0 ± 0,0 gh
	Ki7	16.0 ± 0,0 ef	20.0 ± 0,0 efg
	Ki8	20.0 ± 0,0 cd	21.0 ± 0,0 ef
	Ki9	22.0 ± 0,0 bc	21.0 ± 0,0 ef
	Ki10	20.0 ± 0,0 cd	18.0 ± 0,0 gh
	Ki11	18.0 ± 0,0 de	20.0 ± 0,0 efg
	Ki12	12.0 ± 0,0 g	14.0 ± 0,0 i
	Ki13	14.0 ± 0,0 fg	16.0 ± 0,0 hi
	Ki14	20.0 ± 0,0 cd	16.0 ± 0,0 hi
	Ki15	15.0 ± 0,0 f	19.0 ± 0,0 fg
	Ki16	15.0 ± 0,7 f	19.0 ± 0,7 fg
Cow meat	V2	22.0 ± 0,7 bc	22.0 ± 0,7 de
	V3	22.0 ± 0,0 bc	28.0 ± 0,0 b
	V4	24.0 ± 1,4 b	15.0 ± 2,1 i
	V5	18.0 ± 0,7 de	28.0 ± 0,7 b
Dry cassava	MaS1	14.0 ± 2,8 fg	10.0 ± 2,8 j
	MaS4	10.0 ± 1,4 h	10.0 ± 1,4 j
Soil samples	SM3	29.0 ± 1,4 a	26.0 ± 1,4 bc
	SM4	20.0 ± 1,4 cd	26.0 ± 0,0 bc
	SM5	22.0 ± 0,7 bc	22.0 ± 0,7 de
	SM6	16.0 ± 1,4 ef	18.0 ± 0,0 gh
	SM7	20.0 ± 0,7 cd	22.0 ± 1,4 de

Values followed with different later in superscript on the same column are statistically different ($P < 0.05$).

those reported by Ogunbanwo et al. (2003) who showed that *L. brevis* excreted other compounds such as bacteriocins that inhibited the growth of pathogens.

As shown in Figure 2 and irrespective of the pathogen, the treatment of LF2, V3, V5 and SM4 cell free supernatants with the proteolytic enzyme, proteinase K, at pH 7 resulted in a significant reduction of the antimicrobial activity with SM4 samples mostly totally neutralised. No significant reduction in antimicrobial activity was observed for SM3 neither against *E. coli* CIP548 nor *S. Enterica* CIP8132. This result suggested that although the 5 strains (LF2, V3, V5, SM3 and SM4) exhibited strong inhibition against *S. enterica* CIP8132 and *E. coli* CIP548, four of them were associated with peptide inhi-biting molecules, usually known as bacteriocin. In addi-tion the half inhibition observed for CFS by LF2 and V3 may suggest the presence of other inhibiting molecules such as H_2O_2 in their respective CFS.

In addition to the effect of proteinase K, it was equally observed that lysozyme significantly induced a reduction in the antimicrobial activity of the CFS. In some cases such as the LF2 CFS, the inhibition of the activity was quite higher than that observed for proteinase K. These results, in conformity with others reported in literature (Upreti and Hinsdill, 1973; Enan et al., 1996), suggested the requirement of a carbohydrate moiety for the biological activity of the bacteriocins in our CFS samples. As observed for the effect of proteinase K, lysozyme has no significant effect on the activity of SM3 suggesting that either the strain do not produced bacteriocins or the bacteriocin present was proteinase-K and lysozyme resistant.

Table 3. Physiological and biochemical characteristics of five selected LAB strains.

Growth selection	LAB STRAINS				
	LF2	V3	V5	SM3	SM4
Growth at 8°C	+	+	+	+	+
Growth in NaCl 6,5%	+	+	+	+	+
Growth at 45°C	-	-	-	-	-
Control at 37°C	++++	+++++	+++++	++++	++++
Production of CO_2 from Glucose	-	-	-	-	-
Production of acid From					
Glycerol	ND	-	ND	-	-
Erythritol	ND	-	ND	-	-
D-Arabinose	ND	-	ND	-	-
L-Arabinose	ND	+	ND	+	+
Ribose	ND	+	ND	+	+
D-Xylose	ND	-	ND	-	-
L-Xylose	ND	-	ND	-	-
Adonitol	ND	-	ND	-	-
-méthyl-D-Xyloside	ND	-	ND	-	-
Galactose	ND	+	ND	+	+
Glucose	ND	+	ND	+	+
Fructose	ND	+	ND	+	+
Mannose	ND	+	ND	+	+
Sorbose	ND	-	ND	-	-
Rhamnose	ND	-	ND	-	-
Dulcitol	ND	-	ND	-	-
Inositol	ND	-	ND	-	-
Mannitol	ND	-	ND	-	-
Sorbitol	ND	-	ND	-	-
-methyl-D-mannoside	ND	-	ND	-	-
-methyl-D-Glucoside	ND	-	ND	-	-
N-acethyl-Glucosamine	ND	+	ND	+	+
Amygdalin	ND	+	ND	+	+
Arbutin	ND	+	ND	+	+
Esculin	ND	+	ND	+	+
Salicin	ND	+	ND	+	+
Cellobiose	ND	+	ND	+	+
Maltose	ND	+	ND	+	+
Lactose	ND	+	ND	+	+
Melibiose	ND	-	ND	-	-
Sucrose	+	+	ND	+	+
Trehalose	ND	-	ND	-	-
Inulin	ND	-	ND	-	-
Melezitose	ND	-	ND	-	-
Raffinose	ND	-	ND	-	-
Starch	ND	-	ND	-	-
Glycogen	ND	-	ND	-	-
Xylitol	ND	-	ND	-	-
Gentiobiose	ND	+	ND	+	+
D-Turanose	ND	-	ND	-	-
D-Lyxose	ND	-	ND	-	-
D-Tagatose	ND	+	ND	+	+
D-Fucose	ND	-	ND	-	-
L-Fucose	ND	-	ND	-	-
D-Arabitol	ND	-	ND	-	-
L-Arabitol	ND	-	ND	-	-
Gluconate	ND	?	ND	?	?
2-Keto-Gluconate	ND	?	ND	?	?
5-Keto-Gluconate	ND	-	ND	-	-

(+) designate growth or positive reaction
(-) designate absence of growth or negative reaction
ND = not determined
? = uncertain

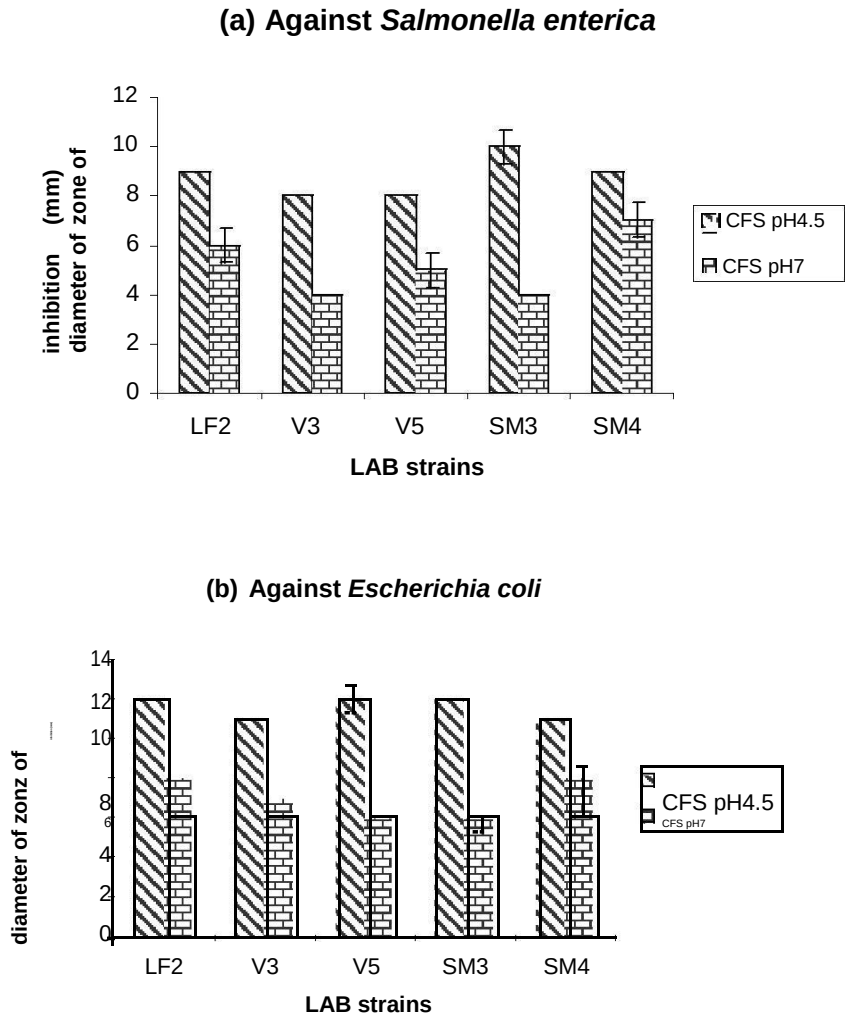

Figure 1. Effect of pH on inhibitory activity against (a) *S. enterica* CIP8132 and (b) *E. coli* CIP548.

In Figure 3, we can see that increase in temperature led to a decrease in antimicrobial activity of most of the CFS samples except that of LF2 which do not varied significantly. This joined our first suggestion made above of the presence of active molecules other than peptides and acids. It is also possible that the bacteriocin produced by the LF2 strain exhibits a thermal stability. This had been demonstrated for bacteriocin produced by *L. brevis* OG1 which showed thermal stability during 60 min at 100°C (Ogunbanwo et al., 2003).

DISCUSSION

The present study reveals that lactic acid bacteria could be readily isolated from all the foods sampled and the bacteriocin producing strains are part of the normal microflora. All of the isolated strains inhibit the growth of the pathogens used as indicators suggesting that antimicrobial lactobacillus are widely present in our environment and more or less naturally protect our foods. Very wide differences were observed on the antimicrobial activity of the 30 isolated lactobacilli strains with 5 strains exhibiting very high inhibition zones (> 25 mm). The difference in the activities of the lactobacilli strains probably reflects the characteristic microflora.

The main objective of this study was to select promising lactobacilli cultures for further study. Many of the bacteriocins gave small inhibition zones, but LF2, V3, V5, SM3 and SM4 gave large zones of inhibition and SM4 had an activity more or less totally inhibited by proteinase K. One strain LF2 was a cocci and produced nisin-like activity. In fact the LF2 bacteriocin could withstand boiling at pH 7.0, and was partially sensitive to proteinase K. These observations are evidence that this strain is producing nisin- like molecule. In addition, the LF2 cultures were able to ferment sucrose, a property that is linked to nisin production on the nisin-sucrose conjugative transposon in lactococci (Rauch et al., 1994).

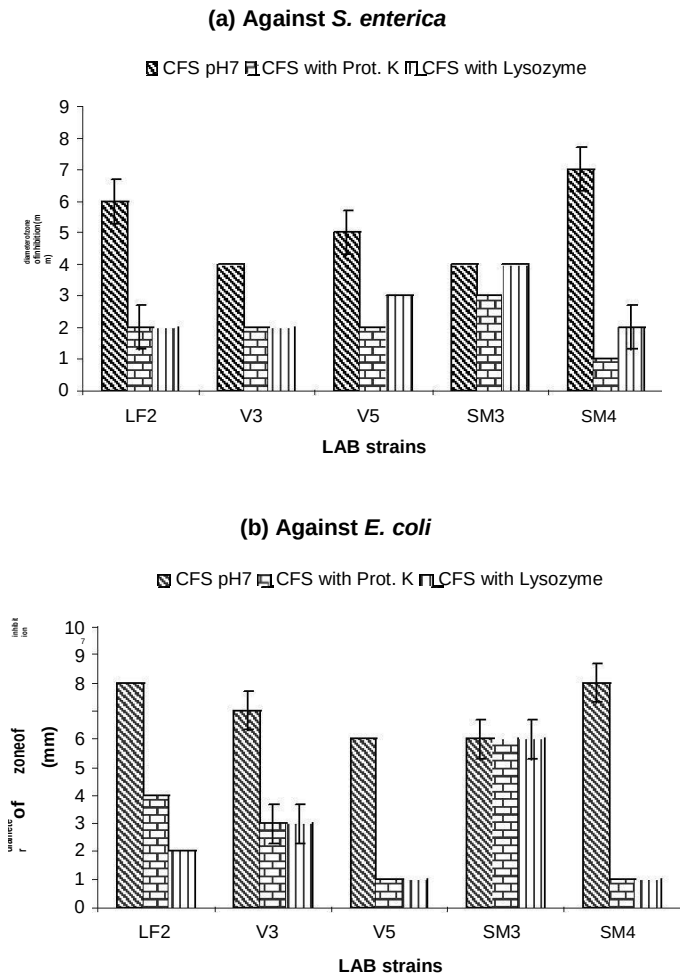

Figure 2. Sensitivity of inhibitory compounds to enzyme activity against (a) *S. enterica* CIP8132 and (b) *E. coli* CIP548

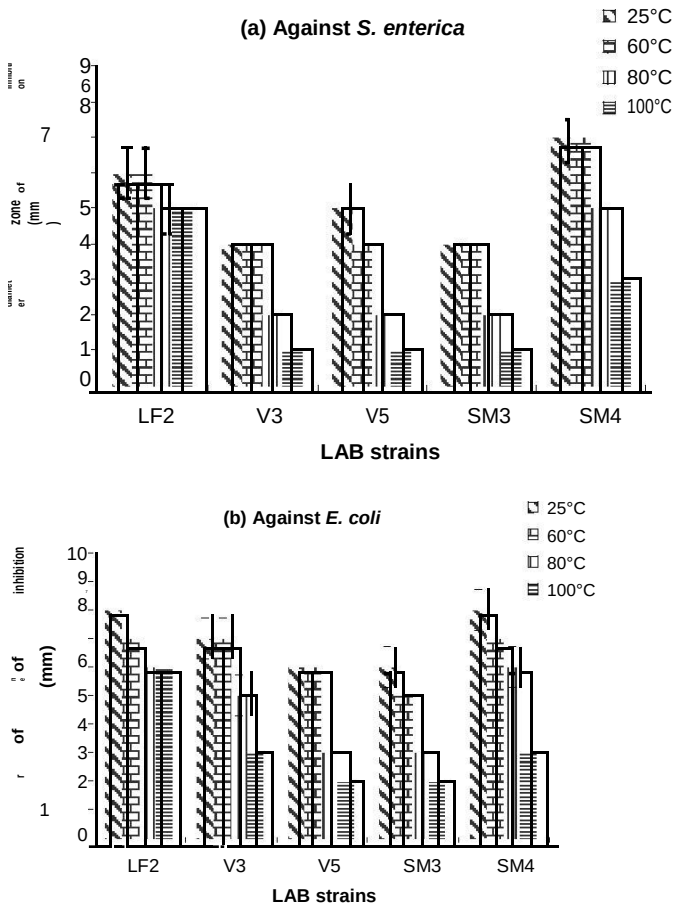

Figure 3. Thermal sensitivity of bacteriocins against (a) *S. enterica* CIP8132 and (b) *E. coli* CIP548.

Lactococci are best studied because of their role as dairy starter cultures, but there have been several reports of strains from non-dairy sources including various vegetable that produce nisin or nisin-like bacteriocins (Harris et al., 1992; Rodriguez et al., 1995; Kelly et al., 1996). In this study strains producing a nisin-like activity was isolated from fresh cow milk. The use of bacteriocins producing cultures as growth factor has potential, especially in poultry where antibiotics use is discouraged.

Conclusion

In conclusion, the present study results on a nisin-like producing lactic acid bacterium isolate from raw cow milk which excellently inhibits the growth of the 2 important chicken's pathogens *S. enterica* CIP8132 and *E. coli* CIP548. This offers many avenues with promising future for further studies towards its use as antimicrobial agent in farming. Future research issues include identification and characterisation of the strain and its bacteriocin and its role as substituent to antibiotics as growth factor in chicken's growth.

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